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Gene expression of components of the insulin/insulin-like signaling pathway in response to heat stress in the garter snake, *Thamnophis elegans*

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The insulin/insulin-like signaling (IIS) pathway is an evolutionary conserved molecular signaling pathway that regulates growth, reproduction, stress resistance, and longevity in response to nutrition and external stress. While the constituents of this pathway and their functions are relatively well understood in laboratory model animals, they have not been explored in many other organisms, with notable exceptions in the fisheries literature. We tested for the gene expression of four key components of this pathway in the garter snake (*Thamnophis elegans*) liver, and determine how the transcription of these components responds to heat stress. We found that the two insulin-like growth factor ligands (IGF-1 and IGF-2) and the receptors (IGF-1 Receptor and M6P/IGF-2 Receptor, or IGF-1R and IGF-2R) are expressed in garter snake liver tissue. Under normal laboratory conditions, IGF-2 and IGF-2R are expressed at a higher level than IGF-1 and IGF-1R. In response to heat stress, IGF-1 expression remained the same, IGF-2 expression decreased, and the expression of both receptors increased. These results demonstrate that elements of the IIS pathway are responsive to heat stress in snakes. Further studies are needed to fully understand the biological consequences of this response.

INDEX DESCRIPTORS: IIS, IGF, reptile, qPCR.

The insulin/insulin-like molecular (IIS) pathway is an evolutionary conserved signaling cascade that responds to nutritional status and external stressors to regulate growth, reproduction, stress resistance, and longevity (Renaville et al. 2002, Kenyon 2010). As part of the somatotropic axis, insulin-like growth factors 1 and 2 (IGF-1 and/or IGF-2) are secreted by the liver (and peripheral tissues) in response to growth hormone (Renaville et al. 2002). Binding of these ligands to the cell membrane-bound IGF-1 Receptor (IGF-1R) initiates a cell signaling cascade that promotes overall cellular and organismal growth and reproduction.

Reduced signaling of the IIS – through, for example, decreased gene expression of IGF ligands and IGF-1R and/or decreased bioavailability of the IGFs to bind the IGF-1R – results in reduces growth and increased longevity and stress resistance (Holzenberger et al. 2003, Muñoz 2003, Cypser et al. 2006). The mannose-6-phosphate/IGF-2 receptor (M6P/IGF-2R) can bind both IGF-1 and IGF-2 ligands, although it preferentially binds IGF-2, causing the ligands to be removed from the bloodstream and targeted for degradation. Thus, M6P/IGF-2R is a negative regulator of the IIS pathway (Denley et al. 2005). Furthermore, IGF binding proteins circulating in the bloodstream modulate the bioavailability of the IGFs to bind the receptors (Firth and Baxter 2002). Known environmental perturbations that affect the expression of these key IGFs signaling proteins include nutrition level (particularly protein) and ambient temperature (Renaville et al. 2002, Gabillard et al. 2003).

Variation in the expression and function of the IIS occurs between groups of animals. In mammals, IGF-2 is an imprinted gene that is highly expressed in the embryo and placenta during development (DeChiara et al. 1991, Constancia et al. 2002), and whose expression declines with age (Li et al. 1996). In fish, both IGF-1 and IGF-2 are expressed in non-embryonic tissues (Gabillard et al. 2003). While M6P/IGF-2R is known to bind IGFs in mammals and some fish, the binding site is missing in chickens and other species of fish (Mendez et al. 2001, Williams et al. 2012). Currently, the gene expression patterns of these IIS genes and the binding of IGF ligands by M6P/IGF-2R in reptiles remain unknown.

Most of what we have learned about the IIS pathway is from lab models (such as nematodes, fruit flies, and mice) and agriculturally important species. Our goal here is to broaden our understanding of the IIS pathway to other species from natural populations who are exposed to widely varying environmental factors, including temperature and nutrition. Using the western terrestrial garter snake, *Thamnophis elegans* – a well-known ecological model organism for studying growth and longevity (Bronikowski 2000, Sparkman et al. 2009) – we evaluated the gene expression of four key components of the IIS pathway using quantitative real-time PCR (qPCR). Specifically, we ask: 1) whether the genes coding for these proteins are expressed in the liver of this reptile, and 2) how the expression of each of these genes responds to a heat stress.

METHODS

Animals

Gravid *T. elegans* females were collected from Eagle Lake in the Sierra Nevada Mountains of California. Females were brought back to Iowa State University to give birth. Offspring were raised in the common laboratory environment for 1.2 yr (juvenile status). Each animal tank had a thermal gradient that ranged from 24°C to 32°C during the day (12 h light), which enabled

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Table 1. qPCR real-time PCR primers. Primers were designed to optimize the following conditions: amplicon length 90 to 150 base pairs; 60°C annealing temperature; GC content of 50%; GC clamp but no more than 3 C or G in last 5 base pairs on 3'end; and to be located over exon-exon boundary to inhibit amplification of contaminating DNA. Modified from Schwartz and Bronikowski (2013).

Gene	Primer Name	Primer sequence (5'-3')	Amplicon length	Tm (°C)	% GC contents	GCs in last 5 bp (3' end)
IGF-1	IGF-1_151.Ex3.qF	GGCCAAGAAACACTTTGTGG	120	60.43	50	3
	IGF-1_247.Ex4.qR	AGTAGAAGAGGAACGCCTACTGC		60.53	52	3
IGF-2	IGF-2_Ex5.qR	TGTTTCTACCCACAGGCCTAC	156	59.11	52	4
	Te_IGF-2_AF	TATTGCTGCTTGTGCTCACC		59.11	52	3
IGF-1R	IGF-1R_Ex13-14.qF	AGTCTGCTGGTTTAGGTTGGAC	132	59.68	50	3
	IGF-1-R_DR	AACTTGCTGGAGCCAAACTG		63.43	50	2
IGF-2R	IGF-2-R_Ex38.qF	CAATAGTGCCATCTCATTTGG	138	58.11	43	2
	IGF-2R_C.3R	GGGAAGACACAAAGCTCACC		59.70	55	3
HSP70	HSP70_1717.qF	AGCACCCTGGTACAATTTGG	133	59.85	50	2
	HSP70_1850.qR	GCTTGAAAAATGCCAAGAGG		59.82	45	3
EEF1a1	EEF1A_Ex5.qF	TGACAAACCTCTACGCTTGC	125	59.08	50	3
	EEF1A_Ex6.qR	TGACTGGAGCAAATGTCACC		59.68	50	3
B-actin	B-actin.Ex3.qF	CCAAGGCCAACAGAGAGAAG	101	59.98	55	2
	B-actin.Ex4.q.R	AGAGGCGTACAGGGAGAGC		59.57	63	3

the animals to behaviorally regulate their body temperature, and 24°C during the night (12 h dark). Animals were fed, 1x per week, thawed mice until satiation, and were last fed 5 days prior to this experiment.

Experimental Design

Animals were split between a standard body temperature (27°C) and a heat stress treatment (37°C). The heat stress temperature of 37°C was chosen to increase their metabolic and oxidative stress (Schwartz and Bronikowski 2013); 37°C is above the preferred body temperature of *T. elegans* (26 to 32°C), but well below the critical thermal max (~43°C) (Stevenson et al. 1985, Huey et al. 1989, Arnold and Peterson 2002). All animals were placed in a 27°C incubator for 20 h to acclimate, and then moved to incubators at their respective temperature treatments (27°C for control and 37°C for heat stress) for 2 h, at which time they were euthanized via decapitation. The liver was dissected out within 10 min of death, and half of the liver was snap-frozen in liquid nitrogen for RNA isolation. Liver was chosen because of its high metabolic activity. RNA was isolated from 12-19 mg of snap-frozen liver from 51 individuals, (24 control, 27 heat stress), using Qiagen RNAeasy kit (Qiagen), with a DNAse digestion on the membrane.

Quantitative real-time PCR

Using quantitative real-time PCR (qPCR) on 51 individuals, we assayed four genes from the IIS pathway (IGF-1 ligand, IGF-2 ligand, IGF-1 Receptor, and M6P/IGF-2 Receptor, hereafter IGF-1, IGF-2, IGF-1R, IGF-2R). Reference genes were the eukaryotic translation elongation factor 1α (EEF1α) and B-actin. We also compared these data to the expression of Heat Shock Factor 70A1 (HSP70) from the same individuals as a positive control, because it is known to increase with heat stress (Mayer and Bukau 2005, Schwartz and Bronikowski 2013). cDNA was synthesized using 1 μg of total RNA, 200 units of SuperScript II reverse transcriptase (Invitrogen), 2 mM deoxynucleotide triphosphates (dNTPs), and 0.5 μg oligo dTs (Invitrogen and Integrated DNA technologies).

Because we were most interested in the relative changes in gene expression in response to heat stress, we use relative quantification based on a standard curve, rather than absolute quantification. A benefit of relative quantification is that we did not need to clone and quantify a PCR insert for each gene; the drawback is that we cannot calculate exact copy number (Pfaffl 2004).

Using a pool of cDNA from all the samples, we created a 10-point standard curve using 1:4 dilutions. For each individual sample, the following dilutions were made from the cDNA: 1:15, 1:50, 1:500, 1:5000. Based on trial runs for each gene, the appropriate set of diluted samples was chosen for each gene, such that the C_T values were between 20 and 30. qPCR reactions were run in duplicate and contained 0.3 μM of each primer (Table 1), and Q-PCR SybrGreen Supermix (Promega), in 20 μL reactions and was conducted on an Eppendorf MasterPlex Cycler using the SybrGreen setting.

The thermal cycle consisted of the following: 95°C for 2 min; 40 cycles of 95°C for 20 sec, 58°C for 20 sec, 68°C for 20 sec; and a final melt curve analysis from 68 to 95°C for 20 min. Individuals that had a C_T standard deviation greater than 0.25 were rerun. A standard curve was run in duplicate on every plate. For every plate, the relevant range of the standard curve (ensuring at least one reference point on either end of the distribution of sample C_T while the curve remained linear) was used to convert C_T values to relative copy number of the transcript for that particular gene.

To test for expression differences between the control and heat stress groups for each candidate gene, we used the REST 2009 Software (Qiagen) (Pfaffl et al. 2002). The method employed by the REST Software takes into account the PCR efficiency for each gene, uses normalization relative to the reference genes (EEF1α and B-actin), and a randomization technique that randomly reallocates control and heat stress samples between the groups to calculate P-values. We used 10,000 randomizations.

RESULTS

All four of the IIS genes were expressed in the liver, although at relatively low levels compared to the HSP70 and the reference

Table 2. qPCR dilution factors and the effect of heat stress on gene expression results for the IIS proteins, HSP70, and the reference genes. The cDNA dilution factors necessary to obtain C_T values between 20 and 30 indicated the relative levels of expression of each gene (lower dilutions indicate lower expression level in the tissue). Fold-change indicates the increase (positive number) or decrease (negative number) in gene expression in response to a 2h heat stress. The - indicates levels were below the threshold for performing an exact test.

Gene	cDNA Dilution	Fold-change
IGF-1	1:15	–
IGF-2	1:50	−0.469 **
IGF-1R	1:15	1.367 *
IGF-2R	1:50	1.538 ***
HSP70	1:5000	274 ***
B-actin	1:500	NA
EEF1a1	1:5000	NA

P-value: * <0.05, ** <0.01, *** <0.001

genes, as determined by the need to use lower dilution factors to obtain C_T values in the range of 20 to 30. IGF-1 and IGF-1R were expressed at the lowest levels (used dilution factors of 1:15); IGF-2 and IGF-2R were expressed at higher levels (used dilution factors of 1:50), the reference and HSP70 genes were expressed at the highest levels (1:500 and 1:5000) (Table 2). In response to heat stress, gene expression of IGF-1 did not change, but IGF-2 was down-regulated (0.5X), and both IGF receptors were up-regulated with heat stress: IGF-1 receptor 1.4X, and M6P/IGF-2 receptor 1.5X (Table 2).

DISCUSSION

Although genes within the insulin/insulin-like growth factor (IIS) pathway are highly conserved across animals, the gene expression patterns are variable. Whereas in mammals, IGF-2 is mainly expressed in embryos and reproductive tissues with decreased expression with age (Li et al. 1996), we found IGF-2 to be expressed at a higher level than IGF-1 in the livers of these juvenile garter snakes, as has been documented in fish (Gabillard et al. 2003). This suggests that IGF-2 may play a more prominent role in the function of the IIS in juvenile and adult reptiles than previously assumed based on what is known in mammals.

The IIS pathway has been implicated as a potential network to modulate cellular protection (promoting longevity) versus growth and reproduction. Previous studies on these garter snakes have demonstrated a correlation between environmental stress in the form of decreased seasonal resource availability and decreased levels of circulating IGF-1 ligand (Sparkman et al. 2009). Here, the qPCR data indicate that the IIS pathway in garter snakes can also be affected by a short-term heat stress that results in a small but significant change in expression, with IGF-2 gene expression being down-regulated, but both receptors up-regulated in the liver.

These results are intriguing and seemingly contradictory at the pathway level. We would predict an increase in M6P/IGF-2R expression to decrease the IIS signaling and promote stress resistance, whereas we would predict an increase in IGF-1R expression to increase the IIS; the biological interpretation of the slight increase in IGF-2 expression is unknown at this time. One important clue may be that the M6P/IGF-2 receptor was originally the mannose 6-phosphate receptor that was co-opted to also bind IGF-2 in mammals and at least one fish (Mendez et al. 2001, Denley et al. 2005), but this binding does not occur in chicken or *Xenopus* (Canfield and Kornfeld 1989, Clairmont and Czech 1989). Thus, these results suggest intriguing possibilities for the role of IGF-2R and IGF-2 in stress response in ectothermic reptiles. However, while it is clear that heat stress affects the gene expression of components of the IIS pathway, the biological implications of these changes are not yet clear.

In summary, we have found that: 1) all four of these key proteins of IIS are expressed in the garter snake liver, and 2) the transcription of specific IIS pathway components responds to short-term heat stress. Certainly, then, pleotropic effects of extreme thermal events on growth, survival, and reproduction in natural populations of ecotherms via the effects on the IIS pathway are plausible. Ongoing studies looking at circulating levels of these growth factors along with transcription levels across tissues will provide additional insight into the role of the IIS pathway in reptiles.

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